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The 3′-to-5′ Exonuclease Activity of Vaccinia Virus DNA Polymerase Is Essential and Plays a Role in Promoting Virus Genetic Recombination▽

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Poxviruses are subjected to extraordinarily high levels of genetic recombination during infection, although the enzymes catalyzing these reactions have never been identified. However, it is clear that virus-encoded DNA polymerases play some unknown yet critical role in virus recombination. Using a novel, antiviral-drug-based strategy to dissect recombination and replication reactions, we now show that the 3′-to-5′ proofreading exonuclease activity of the viral DNA polymerase plays a key role in promoting recombination reactions. Linear DNA substrates were prepared containing the dCMP analog cidofovir (CDV) incorporated into the 3′ ends of the molecules. The drug blocked the formation of concatemeric recombinant molecules in vitro in a process that was catalyzed by the proofreading activity of vaccinia virus DNA polymerase. Recombinant formation was also blocked when CDV-containing recombination substrates were transfected into cells infected with wild-type vaccinia virus. These inhibitory effects could be overcome if CDV-containing substrates were transfected into cells infected with CDV-resistant (CDV^r) viruses, but only when resistance was linked to an A314T substitution mutation mapping within the 3′-to-5′ exonuclease domain of the viral polymerase. Viruses encoding a CDV^r mutation in the polymerase domain still exhibited a CDV-induced recombination deficiency. The A314T substitution also enhanced the enzyme's capacity to excise CDV molecules from the 3′ ends of duplex DNA and to recombine these DNAs in vitro, as judged from experiments using purified mutant DNA polymerase. The 3′-to-5′ exonuclease activity appears to be an essential virus function, and our results suggest that this might be because poxviruses use it to promote genetic exchange.

Genetic recombination is thought to have played a critical role in the evolution of viruses and likely contributes to the dynamics of drug resistance and virulence in viral populations (2, 48). The *Poxviridae* are a clinically important family of viruses that continue to affect human health despite the eradication of variola virus (the causative agent of smallpox) (30, 38, 56). For decades, researchers have taken advantage of the high frequencies of recombination in poxvirus-infected cells to manipulate and study the genetic properties of these pathogens for use as vaccine vectors and oncolytic agents (20, 49). However, surprisingly little is known regarding the mechanism of recombination catalyzed by these (and in fact most other) large DNA viruses.

Poxviruses contain large (~200-kb) DNA genomes that are replicated exclusively in the cytoplasm of infected cells. The prototypic poxvirus, vaccinia virus (VAC), is thought to encode most, if not all, of the genes required for genome replication (55). Poxviruses thus differ from viruses that replicate in the nucleus, such as herpesviruses, which may use both host and viral proteins for DNA replication, recombination, and repair (8, 13, 45, 59, 62). DNA transfection studies have shown that replicating poxviruses catalyze high frequencies of recombination in *trans* and promote double-strand break repair reactions requiring only 15 to 20 bp of end sequence homology (15, 64). During poxvirus replication, large amounts of heteroduplex DNA are formed and resolved, suggesting that single-strand annealing (SSA) reactions may be used for recombinant production during infection (17). An unusual feature of the poxvirus recombination machinery is that ~80% of DNA molecules involved in these duplex-joining reactions are first processed in a 3′-to-5′ manner in vivo (63, 64), whereas duplex ends are typically processed in a 5′-to-3′ direction in other viral and cellular homologous recombination systems (7, 23, 44, 58).

Neither classical genetic methods nor bioinformatic approaches have directly identified the VAC gene product(s) catalyzing these reactions. However, it has been noted that recombination becomes temperature sensitive in cells infected with VAC strains carrying temperature-sensitive mutations in the virus *E9L* (DNA polymerase) gene (6, 41, 60) and is also suppressed by DNA polymerase inhibitors (6, 15). These observations suggested that poxviral DNA polymerases play some role in promoting virus recombination. What that role might be cannot be determined a priori but could involve the production of broken replication forks after polymerase stalling, processing/extension of DNA strands during SSA, and/or postsynaptic DNA repair.

The possibility that poxvirus DNA polymerases might function in directly catalyzing virus recombination was first suggested when VAC DNA polymerase (E9) activity was found to copurify with an induced strand transfer activity from infected cells (60). Later studies have shown that the 3′-to-5′ exonuclease (proofreading) activity of highly purified E9 can also catalyze the fusion of linear DNA duplexes into joint molecules (61). These duplex-strand-joining reactions require limited sequence homology (≥12 bp), are stimulated by the virus-en-

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coded single-stranded DNA-binding protein (I3), and require the 3′-to-5′ exonuclease activity of E9 (61). Evidence for the requirement for the E9-encoded 3′-to-5′ exonuclease activity in these reactions comes from the observations that 5′ ^{32}P labels, but not 3′ ^{32}P labels, are retained in reaction products, that these reactions require Mg^{2+}, and that joint-molecule formation is inhibited by high deoxynucleoside triphosphate (dNTP) concentrations (22, 61). Collectively, these data suggested that poxviruses might employ an unusual recombination system in which the E9-encoded 3′-to-5′ exonuclease activity plays a role in resecting broken DNAs, generating 5′-ended single-stranded tails that can then anneal with other complementary single-stranded DNAs. Furthermore, our previous in vitro studies found that VAC E9 efficiently repairs gaps or 3′ flaps that are typical of "imperfect" recombinant intermediates after SSA, suggesting that poxvirus polymerases may also be involved in the postsynaptic events of SSA (21).

Despite the many complementary pieces of biochemical and genetic evidence implicating the polymerase's 3′-to-5′ exonuclease in catalyzing viral recombination, a clear link had yet to be demonstrated in vivo. Ideally, one would use viruses bearing a deficiency in the E9-encoded 3′-to-5′ exonuclease activity to study this phenomenon and thus avoid the lethal consequences of ablating the 5′-to-3′ DNA polymerase activity. Unfortunately, as we report in this communication, the E9-encoded exonuclease appears to be essential for virus viability. However, we have developed a novel, antiviral-based strategy for the study of the role of E9 in viral recombination. This approach was developed from our previous studies of the mechanism of action of the dCMP analog cidofovir {(*S*)-1-[3-hydroxy-2-(phosphonylmethoxypropyl) cytosine]} (CDV) (34, 35) as well as through the characterization of CDV-resistant (CDV^r) VAC strains (1).

We have previously shown that VAC DNA polymerase can use the diphosphoryl metabolite of CDV (CDVpp) as a substrate and faithfully incorporate a CDV residue opposite dGMP in the template strand (35). Importantly, when CDV is in the penultimate position in the primer strand, it creates a primer terminus that is both poorly extended and highly resistant to E9 proofreading activity (35). Furthermore, when CDV is present in the template strand, it blocks translesion DNA synthesis by E9 (34). These studies suggested that CDV^r viral DNA polymerases would have to avoid incorporating these drugs into DNA and/or would have to accommodate the molecules of drug that do get into the template strand in order to generate resistance.

We have recently isolated CDV^r VAC after repeated passage of wild-type virus in media containing escalating doses of CDV (1). These mutant viruses encoded two amino acid substitutions in portions of E9 (and in other DNA polymerases) containing the exonuclease (A314T) and polymerase (A684V) domains. The two different map positions of these mutations in *E9L*, the striking differences in the profiles of cross-resistance to other DNA polymerase inhibitors between strains encoding each single mutation, and the observation that viruses encoding both mutations are ~5-fold more CDV^r than either single mutant strain suggested that these mutations act independently (1). These observations led us to speculate that the A314T substitution might primarily affect 3′-to-5′ excision of CDV, while the A684V change might primarily affect either the enzyme's capacity to discriminate against CDVpp as a substrate or its capacity to accommodate templates bearing CDV.

In this communication we show that a VAC DNA polymerase bearing the A314T substitution can overcome the inhibitory effects of CDV in both in vitro recombination and exonuclease assays. Furthermore, viruses encoding this substitution also promote CDV^r recombination in vivo. Viruses encoding only the A684V substitution, however, do not exhibit this phenotype. We also provide further data and a model for poxvirus recombination based on 3′-to-5′ exonuclease processing of recombination substrates by E9, followed by SSA, that is enhanced by viral I3 and modulated by dNTP pools. To our knowledge, both the polarity of end resection and the involvement of the viral DNA polymerase proofreading activity establish this mechanism as unique among homologous recombination schemes.

MATERIALS AND METHODS

Cell and virus culture. The cell and virus culture methods used in this study have been described elsewhere (1). Wild-type VAC and its CDV^r derivatives were obtained as described previously (1) and are derived from a stock of VAC (strain WR) originally acquired from the American Type Culture Collection. Viruses carrying temperature-sensitive mutations in the *E9L* gene (D*ts*83 and C*ts*42) were obtained from R. Condit (Gainesville, FL). Cells and viruses were normally cultured in modified Eagle's medium (MEM) supplemented with 5% fetal bovine serum. However, cells were cultured in Opti-MEM (Invitrogen) in experiments requiring transfections. All of the cells used in this study tested negative for mycoplasma.

Drugs and proteins. CDV and CDVpp were gifts from K. Hostetler (San Diego, CA), and hydroxyurea (HU) was purchased from Boehringer (Indianapolis, IN). Wild-type VAC DNA polymerase was prepared using a VAC expression system (37). The A314T mutant DNA polymerase was prepared by infecting 200 150-mm-diameter dishes of BSC-40 cells with VAC strain V-DG314-5 (1) at a multiplicity of infection of 10. The cells were harvested and the enzyme purified using methods identical to those described previously (37). Silver-stained gels showed that the mutant enzyme was comparable in purity to wild-type preparations. The enzymes were stored at −20°C in glycerol and were freshly diluted in a buffer containing 25 mM potassium phosphate (pH 7.4), 5 mM β-mercaptoethanol, 1 mM EDTA, 10% (vol/vol) glycerol, and 0.1 mg/ml bovine serum albumin prior to use. The recombinant VAC single-stranded DNA-binding protein I3 was prepared as described previously (53).

DNA substrates and site-directed mutagenesis. The DNA substrates used in recombination assays have been described elsewhere (64). Plasmid pRP406 harbors a luciferase gene driven by a VAC P11K promoter, and pRP7.5*lacZ* harbors a β-galactosidase gene under the control of a VAC P7.5 promoter (64). Plasmid pBluescript II KS was obtained from laboratory stocks. Plasmids pRP406Δ and pRP403Δ are derived from pRP406 and share 366 bp of overlapping homology between upstream and downstream portions of the luciferase open reading frame (ORF). Recombination between pRP406Δ and pRP403Δ reconstructs the luciferase ORF (64).

Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (Strategene, TX). Several different primer sets were used to separately introduce D-to-A substitution mutations (either D166A, D268A, or D462A) into full-length cloned copies of the *E9L* gene along with closely linked restriction site polymorphisms (Table 1). Three additional primer sets were used to introduce variant restriction sites into the *E9L* gene without altering D166, D268, or D462.

Preparation of CDV-containing DNA substrates. Linear substrates were prepared by digesting pBluescript with XhoI or SpeI and pRP406 with XhoI or AflIII. The DNA was gel purified using a Qiaquick kit (Qiagen), and then 3 pmol of each DNA was incubated with 5 ng/μl of VAC DNA polymerase in a 20-μl reaction mixture containing 0.4 mM dNTPs and a "polymerase reaction buffer" comprising 30 mM Tris-HCl (pH 7.9), 5 mM $MgCl_2$, 70 mM NaCl, 1.8 mM dithiothreitol, and 88 μg/ml bovine serum albumin (35). Where noted, dCTP was replaced with CDVpp. The reaction mixtures were incubated for 15 min at 37°C; reactions were stopped by the addition of EDTA; and products were purified using a G-50 spin column (Amersham) followed by a Qiaquick spin column. The

TABLE 1. Marker rescue strategy to rescue VAC encoding E9 3′-to-5′ exonuclease-inactivating substitutions

Motif	Coding strand sequence[a]	Allele	Revertants[b] at 39.5°C	Restriction profile[c]
Exo I	P R S Y L F L **D** I E C H F CCCAAGATCGTACTTATTTCTAG**A**TATAGAGTGTCACTTCG	Parental/target	N/A	N/A
	P R S Y L F L **A** I E C H F CCCAAGATCGTACTTATTTCT**AG**C**T**ATAGAGTGTCACTTCG AluI	D166A mutant	Yes	Parental
	P R S Y L F L **D** I E C H F CCCAAGATCGTACTTATTTCTAG**A**CATAGAGTGTCACTTCG	Wild-type control	Yes	Parental
Exo II	V V T F N G H N F **D** L R Y I T N GTCGTTACCTTTAACGGACATAACTTTG**A**TCTG**A**GATATATTACTAATC DdeI	Parental/target	N/A	N/A
	V V T F N G H N F **A** L R Y I T N GTCGTTACCTTTAACGGACATAACTTTG**C**TCTG**C**GATATATTACTAATC	D268A mutant	Yes	Parental
	V V T F N G H N F **D** L R Y I T N GTCGTTACCTTTAACGGACATAACTTTG**A**TCTG**C**GATATATTACTAATC	Wild-type control	Yes	Parental + control
Exo III	A R Y C I H **D** A C L C N Y L W GGCTAGATACTGTATTCATG**A**TGC**T**TGTTTGTGTCAGTATTTGTGG BspHI	Parental/target	N/A	N/A
	A R Y C I H **A** A C L C N Y L W GGCTAGATACTGTATTCATG**C**TGC**A**TGTTTGTGTCAGTATTTGTGG	D462A mutant	Yes	Parental
	A R Y C I H **D** A C L C N Y L W GGCTAGATACTGTATTCATG**A**TGC**A**TGTTTGTGTCAGTATTTGTGG BspHI NsiI	Wild-type control	Yes	Parental + control

[a] Gene sequence surrounding each of the three indicated exonuclease motifs. For each motif, the sequence of the D*ts*83 or C*ts*42 parent strain (the parent/target allele) is shown at the top, the sequence of a transfected DNA designed to introduce a substitution mutation at invariant aspartic acid residues (boldface) (D166A, D268A, or D462A mutant allele) is shown in the middle, and the sequence of a transfected control DNA, designed to create closely linked but genetically silent sequence polymorphisms (wild-type control allele) is shown at the bottom. Also shown are the restriction sites that may be added or ablated through recombination. The DNA sequences shown are the forward primers used (in conjunction with complementary reverse primers) in the site-directed mutagenesis step that was used to generate the six different transfected *E9L* genes.

[b] Virus-infected cells were transfected with either mutant or control *E9L* alleles and left to replicate for 24 h at 31.5°C. The titers of the virus progeny were determined at 31.5°C. The viruses were then replated on BSC-40 cells at 39.5°C (10,000 PFU/60-mm-diameter dish) and cultured 1 to 2 days, and any plaques were stained with crystal violet. No plaques were recovered at 39.5°C if DNA was omitted from the transfection mixture.

[c] A 3.1-kb fragment carrying the *E9L* gene was amplified by PCR from DNA extracted from cells infected with recombinant VAC capable of replicating at 39.5°C. The DNA was digested with AluI, DdeI, or BspHI (or NsiI) in order to look for polymorphic restriction sites linked to Exo I, Exo II, or Exo III mutations, respectively (see Fig. 1).

DNA was quantified by spectrophotometry and stored at −20°C. Labeled substrates were prepared in the same way except that reaction mixtures contained 10 μM cold dTTP supplemented with 50 μCi of [α-^{32}P]dTTP (Amersham).

To test the efficiency of these "end-filling" reactions, three different oligonucleotide duplexes, each consisting of an 18-base primer annealed to a 22-base template, were prepared. Each encoded the 4-base 3′ recessed end generated using the XhoI, SpeI, or AflIII restriction enzyme. The XhoI substrate comprised primer P.1 (5′-TGACCATGTAACAGAGAC-3′) annealed to template T.1 (5′-TCGAGTCTCTGTTACATGGTCA-3′); the SpeI substrate comprised P.2 (5′-TGACCATGTAACAGAGAA-3′) and T.2 (5′-CTAGTTCTCTGTTACATGG TCA-3′); and the AflIII substrate comprised P.2 (above) and T.3 (5′-CGTGTT CTCTGTTACATGGTCA-3′). Before use, each primer strand was 5′ end labeled with polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP (Amersham). These substrates were incubated for 15 min with VAC DNA polymerase as described above, and the reactions were stopped by the addition of 10 μl of a formamide-containing gel-loading buffer. The products were then size fractionated using a 10% polyacrylamide sequencing gel, fixed, dried, and imaged using a Typhoon phosphorimager (35).

In vitro duplex-joining and exonuclease assays. The duplex-joining assay has been described elsewhere (61). Briefly, each 20-μl reaction mixture contained 2.5 ng/μl of either wild-type or mutant VAC DNA polymerase, 25 μg/ml of the VAC single-stranded DNA-binding protein I3, 300 ng of SpeI-cut and XhoI-cut pBluescript DNA (filled in as described above), and the polymerase reaction buffer. The reaction mixtures were incubated at 37°C; reactions were stopped with EDTA; the products were deproteinized; and the DNAs were separated using a 1% agarose gel. The DNA was stained with ethidium bromide, and the band intensities were determined using a Gel Logic 200 imager and Kodak 1D software.

Each 30-μl exonuclease assay mixture contained 3.3 ng/μl of either wild-type or mutant VAC DNA polymerase, 300 ng (~30,000 cpm) of ^{32}P-labeled, XhoI-cut pBluescript DNA (filled in as described above), and the polymerase reaction buffer. The reaction mixtures were incubated at 37°C, and reactions were stopped when noted by the addition of 6 μl of 0.5 M EDTA and 30 μl of 0.1 M sodium pyrophosphate. The amount of trichloroacetic acid-soluble radioactivity was then determined using an LS 6500 liquid scintillation counter (Beckman Coulter).

In vivo recombination assays. We used a transfection-based recombination assay that has been described elsewhere (64). Briefly, 35-mm-diameter dishes of BSC-40 cells were infected with VAC at a multiplicity of infection of 2, and the cells were then transfected with 100 ng of each of the two indicated recombination substrates plus 100 ng of pRP7.5*lacZ* by using Lipofectamine 2000 (Invitrogen). Protein extracts were prepared when indicated, and the amounts of luciferase and β-galactosidase were determined by using luciferase (Promega) and β-galactosidase (Clontech) luminescence detection kits. The amount of β-galactosidase activity detected was used to correct for variations in transfection efficiencies. The recombinant frequency (Rf) was calculated from the normalized amount of luciferase detected in cells cotransfected with pRP406Δ and pRP403Δ relative to the normalized amount of luciferase detected in cells transfected in parallel with pRP406, by using the formula Rf = 100% × $[(\text{Luc/LacZ})_{\text{pRP406}\Delta+\text{pRP403}\Delta}]/[(\text{Luc/LacZ})_{\text{pRP406}}]$ (64). Each measurement was an average calculated from three dishes, and at least three independent experiments were performed. Where indicated, CDV was added to the culture medium 24 h prior to infection and again 1 h postinfection at a concentration equal to the 50% effective concentration (EC_{50}) for each virus. The EC_{50} represents the drug concentration at which each virus generates 50% of the progeny that are produced in the absence of the drug. The EC_{50}s are 53 μM for wild-type VAC and 240, 140, and 890 μM for VACs encoding the A314T, A684V, and A314T A684V mutations, respectively (1). Where indicated, HU-containing medium was added 4 h postinfection (50).

Southern and slot blotting. Southern blotting was performed using standard methods (64). Briefly, 60-mm-diameter dishes of BSC-40 cells were infected with VAC for 1 h at a multiplicity of infection of 5 and were then transfected with 200

ng of each indicated DNA. Total cellular DNA was recovered 24 h postinfection, cut with MbiI (and with DpnI to degrade unreplicated input DNA), sized using a 1% agarose gel, and transferred to a nylon membrane (Bio-Rad). The DNA was hybridized to a ^{32}P-labeled luciferase gene probe, and the bound label was detected and analyzed using a phosphorimager and ImageQuant software (version 5.1). Slot blots were used to measure the amount of virus DNA replication in cells transfected with small interfering RNAs (siRNAs) targeting *I3L* mRNA. DNA was recovered from infected cells that had been treated with siRNA as described in the next section, spotted onto a nylon membrane (Bio-Rad), hybridized to a ^{32}P-labeled *E9L* gene probe, and imaged as described for Southern blotting.

siRNA treatment and Western blotting. BSC-40 cells were cultured in 35-mm-diameter dishes and transfected, using Lipofectamine 2000, with 100 pmol/dish of a siRNA targeting *I3L* mRNA. Several siRNAs were designed and synthesized by Qiagen, and one, designated *I3L*-4 (target, 5′-AAGGAGAGACUAAACUU UAUA-3′), was found to produce the highest levels of I3 knockdown. AllStars control siRNA (Qiagen) was used as a negative control for I3 knockdown. Twenty-four hours later, these cells were infected with virus and transfected with the recombination substrates as described above. An additional 100 pmol/dish of each indicated siRNA was added along with the DNA.

Cell extracts were prepared in duplicate at 8 h postinfection, with one aliquot being used for luciferase assays. A second aliquot of protein was harvested using 250 μl of radioimmunoprecipitation assay cell lysis buffer, size fractionated using a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel, and then transferred to a nitrocellulose membrane. The blot was hybridized to a mixture of primary antibodies targeting I3 (31) (1:5,000-diluted mouse monoclonal antibody) and β-actin (1:10,000-diluted mouse monoclonal antibody; Sigma) and was then probed with a secondary antibody bearing an infrared dye (1:20,000-diluted goat anti-mouse antibody; Li-Cor) by using buffers and methods as directed by the supplier. The blots were imaged using a Li-Cor Odyssey scanner.

Statistical analyses. Statistical analyses were conducted where at least four independent experiments had been performed. Although means are presented for simplicity, we used more conservative, nonparametric statistical tests to compare median Rfs between treatments. In cases where Rfs, expressed as a percentage, were analyzed, each value was first divided by 100 and then logit transformed. In Fig. 2B and 5D, the median Rfs for control and CDV treatments within each virus type were compared using Mann-Whitney U tests. For Fig. 8, median Rfs for each drug concentration were first analyzed by a Kruskal-Wallis test, followed by a Dunn multiple-comparison posttest, to determine which pairs of treatments were significantly different ($P < 0.05$). All statistical analyses used GraphPad Prism (San Diego, CA) software (version 4.0).

RESULTS

The VAC *E9L*-encoded 3′-to-5′ exonuclease appears to be essential for virus viability. Studies with phage T4 and herpes simplex virus have shown that one can inactivate the DNA polymerase proofreading activity and retain viable virus (18, 27). This could provide a route for testing the role of the VAC E9 3′-to-5′ exonuclease in recombination while avoiding the lethal effects on replication of inactivating the DNA polymerase completely. To test this, we used a marker rescue scheme and a selection strategy based on the reversion of closely linked temperature-sensitive mutations in the *E9L* gene. The VAC strains encoding these mutations replicate normally at 31.5°C but do not replicate at 39.5°C (29). BSC-40 cells were infected for 1 h at 31.5°C with VACs encoding point mutations in the 3′-to-5′ exonuclease (*E9L* allele D*ts*83 [H185Y]) or the 5′-to-3′ polymerase (*E9L* allele C*ts*42 [E611K]) domain (29) (Fig. 1A). After 1 h of infection, the cells were transfected with DNA encoding wild-type sequences at the two temperature-sensitive allele sites but containing one of three different mutations in the E9 exonuclease domain (D166A, D268A, or D462A) (Fig. 1A and Table 1). These three aspartic acid residues are highly conserved in other B-family DNA polymerases, and the D-to-A substitutions are known to inactivate exonuclease function (3, 14). Each of these mutations also modified, or was closely linked to, a polymorphic restriction site marker. As a control, cells were also transfected with *E9L* alleles that encoded wild-type sequences at the two temperature-sensitive sites but encoded silent mutations at (or very near) one of the aspartic acid-encoding codons mentioned above (Table 1). The infected and transfected cells were cultured for 24 h at 31.5°C and then subjected to freeze-thawing, and the viral progeny were plated on fresh cells and cultured at 39.5°C for another 1 to 2 days to select for recombinant, temperature-resistant virus. Virus DNA was isolated from the cultures grown at 39.5°C, both in bulk and from individual plaques, and the *E9L* genes were PCR amplified and then screened for the presence of any of the three linked exonuclease-inactivating mutations by using the linked restriction site polymorphisms. In parallel, we determined the yields of viruses of all genotypes, from infected and transfected cells, by using plaque assays performed at 31.5°C.

Recombinant viruses were recovered from all of the transfected cell cultures. However, a striking feature of the data was that the closer the D-to-A substitution was located to the reverted temperature-sensitive allele, the lower the frequency of recovery of temperature-resistant recombinants. For example, the yield of recombinant viruses dropped ~5-fold when we attempted to revert the D*ts*83 (H185Y) temperature-sensitive marker with an *E9L* gene encoding the D166A mutation rather than the D462A mutation (Fig. 1B). No such effects were seen in cells transfected with control DNAs encoding only silent restriction site polymorphic markers (Table 1 and Fig. 1B). To test whether the few revertant viruses still encoded the desired exonuclease mutations, we extracted DNA from cells infected with the temperature-resistant virus (either in bulk or plaque purified), amplified the *E9L* locus by PCR, and digested the products with restriction enzymes targeting polymorphic sites located very close (≤5 nucleotides [nt]) to each of the three exonuclease mutations (Fig. 1C to F). In none of these experiments did we detect virus DNA encoding a restriction pattern diagnostic for a virus encoding a D-to-A substitution in any of the three exonuclease motifs. For example, the D166A mutation would create an AluI site (Table 1) readily detectable in the input DNA used for transfection but absent in DNA extracted from a pool of DNA amplified from the temperature-resistant virus (Fig. 1C). Similarly, the DNA used to introduce the D268A mutation (as well as the corresponding control DNA) was designed to also silently ablate a nearby DdeI site (Table 1). None of the temperature-resistant viruses recovered from cells transfected with D268A mutant DNA encoded a disrupted DdeI site, whereas we had no difficulty disrupting the site if we transfected a control DNA encoding the wild-type aspartic acid residue (Fig. 1D). The same effects were seen in attempts directed at mutating the Exo III motif (Fig. 1E and F) and using the *E9L* C*ts*42 mutation as an alternative selectable target (data not shown). Thus, we have never been able both to revert the temperature-sensitive phenotype and to introduce an exonuclease-inactivating mutation. These results suggest that one cannot, under these conditions, generate a VAC strain lacking the *E9L*-encoded proofreading function.

Effect of CDV on circle-by-circle recombination in vivo. As noted above, the use of the antiviral drug CDV and CDV^r VAC strains may provide an alternative approach to studying the role of E9 in viral recombination. To test this hypothesis,

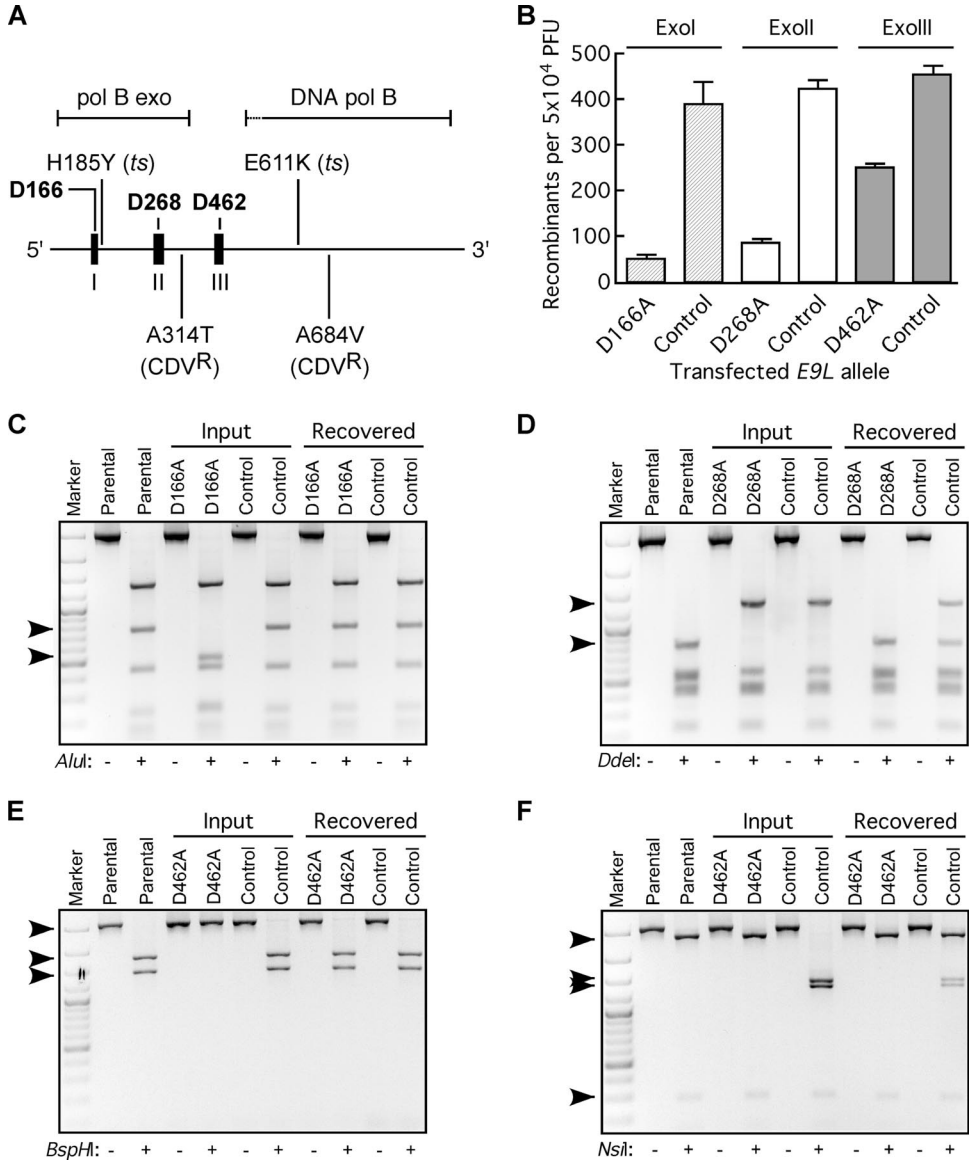

FIG. 1. Characterization of mutant and recombinant vaccinia viruses. (A) Map of the VAC *E9L* gene. The *E9L* gene spans 1,006 amino acids and comprises 3′-to-5′ exonuclease (pol B exo) and 5′-to-3′ polymerase (DNA pol B) domains (36). The 5′ end of the gene encodes three highly conserved exonuclease motifs (I, II, and II), each of which includes an aspartic acid residue that is predicted to be essential for exonuclease activity (D166, D268, and D462). Also shown are the map positions of two temperature-sensitive mutations and two CDV^r alleles used in this study. (B) Effect of selection for exonuclease mutations on the recovery of recombinant virus in BSC-40 cells. Cells were first infected with VAC encoding the D*ts*83 (H185Y) allele at 31.5°C and then transfected with DNAs encoding the indicated D-to-A substitution alleles or related control DNAs (see Table 1). The titers of the progeny were determined at 31.5°C and 39.5°C in order to ascertain the relative yields (means ± standard deviations) of recombinant (i.e., temperature-resistant) viruses. (C to F) Genotyping of recombinant viruses. PCR was used to amplify a 3.1-kb fragment of the *E9L* gene by using as the template either a virus carrying the D*ts*83 allele ("Parental"), the indicated mutant or control DNA that was transfected into D*ts*83-infected cells ("Input"), or DNA extracted from cells infected with a mixture of temperature-resistant progeny viruses ("Recovered"). The PCR amplicons were digested with the indicated restriction enzymes, and the presence or absence of each site was used to differentiate the genotypes. For example, none of the recombinants recovered from cells transfected with DNA carrying the D166A allele inherit the AluI site that would be created by this mutation (C). Thus, although all of the viruses are recombinant at the D*ts*83 locus, none encode an ExoI mutation. See the text and Table 1 for additional discussion. Arrowheads indicate the positions of diagnostic restriction fragments, which serve to differentiate *E9L* alleles.

we used an infection-and-transfection scheme, which detects the reconstruction of a luciferase reporter gene through recombination between two plasmids sharing 366 bp of luciferase gene homology (pRP403Δ and pRP406Δ) (64). This method permits the detection of recombinants by either Southern blotting (Fig. 2A) or enzymatic assays (Fig. 2B). In control experiments, BSC-40 cells were infected with VAC and then cotransfected with pRP403Δ and/or pRP406Δ. Southern blot analysis showed that ~15% of the DNA could be recovered in a recombinant form under these circumstances, whereas trans-

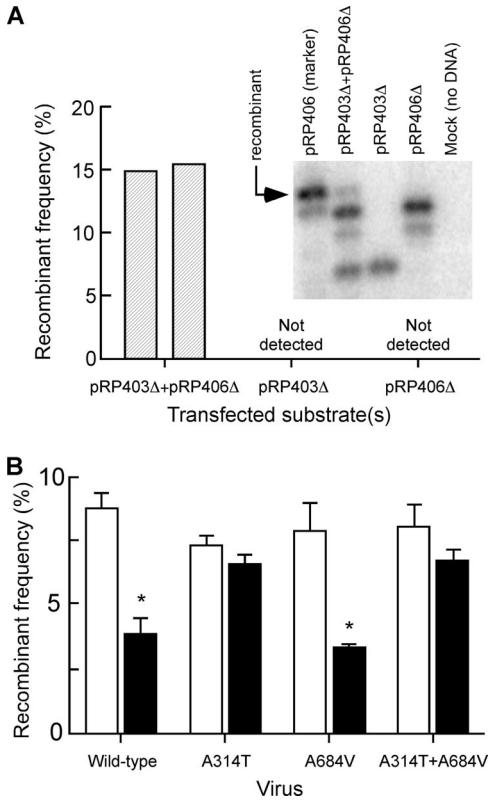

FIG. 2. Effect of CDV on plasmid-by-plasmid recombination in VAC-infected cells. (A) Southern blot analysis of recombinant molecules isolated from wild-type VAC-infected cells. VAC-infected cells were transfected with the indicated circular plasmid DNAs, and then the DNA was harvested 24 h postinfection. The DNA was digested with MbiI and DpnI, size fractionated, and hybridized to a ^{32}P-labeled luciferase probe, and plasmid products were detected by phosphorimaging. Recombination is expected to produce a 2.5-kb recombinant band (arrow). The Rf was calculated by dividing the intensity of the recombinant band shown in an experimental lane by the intensity of the 2.5-kb band recovered from cells transfected with a plasmid encoding a full-length luciferase gene (pRP406). The bar graph shows the results of two independent experiments, and the inset shows one of the blots. (B) Luciferase-based detection of plasmid-by-plasmid recombination in the presence (filled bars) or absence (open bars) of CDV. The cells were exposed to a CDV-containing medium for 24 h prior to infection with the indicated VAC strains, using the drug doses described in Materials and Methods. The cells were infected for 1 h and then transfected with pRP403Δ and pRP406Δ in a drug-free or CDV-containing medium. The CDV^r VAC strains are indicated by the E9 amino acid substitution(s) encoded. Protein extracts were prepared 6 h postinfection, and the Rf was calculated from the amount of luciferase activity as described in Materials and Methods. The mean Rfs (expressed as percentages) are shown in the bar graph. Error bars indicate the SEs of the means for four independent experiments. An asterisk indicates a significant ($P < 0.05$) difference between control and CDV-treated cells for a VAC strain.

fection of either of these substrates alone did not produce detectable recombinants.

After confirming that these assays were working as had been previously demonstrated (64), we examined what effect CDV would have on these reactions. BSC-40 cells were infected with either wild-type VAC or one of three different CDV^r VAC strains encoding the A314T, A684V, or A314T A684V *E9L* substitution mutations (1) and were then transfected with pRP403Δ and/or pRP406Δ. We have previously shown that these mutant viruses replicate to titers indistinguishable from those of wild-type VAC in the absence of CDV while demonstrating profound differences in replication in the presence of the drug (1). This creates a practical problem, because if a particular virus strain cannot replicate the transfected DNA, one has no way of monitoring recombinant production. Therefore, in order to place these viruses under similar degrees of drug pressure and thus ensure similar levels of replication across all of the experiments, we exposed each of the virus strains to a CDV concentration equal to the EC_{50} for that virus on BSC-40 cells (see Materials and Methods). Using the enzymatic version of the assay, we observed that each VAC strain exhibited a similar mean Rf, in the absence of drug, of ~8% (Fig. 2B). However, when CDV was added throughout the course of the experiment, it caused a significant ($P < 0.05$) reduction in the Rf in cells infected with the wild-type or A684V-encoding virus, from 8.8% ± 0.6% (mean ± standard error [SE]) to 3.9% ± 0.6% and from 7.9% ± 1.1% to 3.4% ± 0.1%, respectively. Interestingly, drug treatment had no significant effect on Rf in cells infected with a virus encoding the A314T exonuclease domain substitution, either alone or in conjunction with the A684V substitution (Fig. 2B). These results show that CDV, like other DNA polymerase inhibitors (6, 15), can inhibit recombination reactions in vivo. However, the effect of the drug depends on the genotype of the virus at the *E9L* locus.

Preparation of CDV-containing linear recombination substrates. The experiments described above showed that E9 plays some role in promoting recombination in VAC-infected cells, since certain *E9L* mutations were linked to a different capacity of the virus to recombine circular substrates in the presence of a DNA polymerase inhibitor. We were uncertain whether the effects we are seeing relate to differences in how the various VAC polymerases use CDVpp as a substrate, replicate DNA strands containing CDV, or excise CDV using 3′-to-5′ proofreading activities. These concerns can be addressed by using, as substrates, linear DNA duplexes bearing CDV incorporated into the penultimate positions of their 3′ ends. These substrates provide a specific tool for inhibiting the activity of the 3′-to-5′ exonuclease of E9 (35) and offer three advantages over the methods used in the preceding experiment. First, linear molecules are recombined more efficiently than are circles in poxvirus-infected cells (64), and thus, this approach provides greater experimental sensitivity. Second, one knows precisely where the CDV is located and need not make any assumptions regarding differential rates of CDV uptake and incorporation into DNA. Finally, poxviruses cannot replicate linearized plasmid DNAs that are transfected into infected cells unless they are first recombined into circles (10, 12, 64). Thus, the system we are studying becomes recombination dependent, since circularization, through recombination, must precede the replication of these DNAs.

To make these substrates, we first cut pBluescript with XhoI or SpeI to create two 3-kbp DNAs that shared 57 bp of overlapping sequence homology. The extent of the overlap significantly exceeds the 15 to 20 bp that we have previously defined as being the minimum required for permitting efficient double-strand break repair in poxvirus-infected cells (64). These enzymes generate a 4-base overhang that contains a dGMP res-

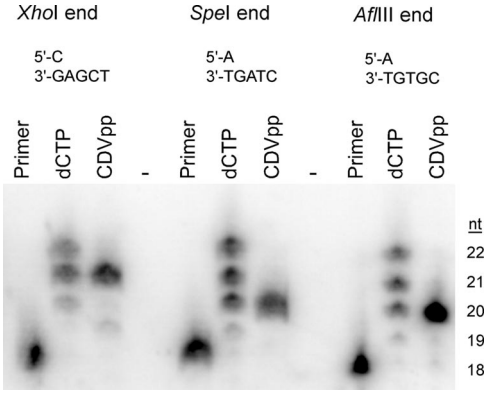

FIG. 3. Characterization of "end-filling" reaction products. Each 18-nt primer was 5′ end labeled with ^{32}P, purified, and annealed to a 22-nt template strand. This created three duplex DNAs bearing the same 3′-recessed ends as those produced by the indicated restriction enzymes. The primer strands were then filled in using VAC DNA polymerase in reaction mixtures containing dGTP, dATP, dTTP, and either dCTP or CDVpp. The reaction products were size fractionated on a denaturing 10% polyacrylamide gel and detected using a phosphorimager. The sizes of the extension products are indicated along with the migration position of each 18-mer ^{32}P-labeled primer strand.

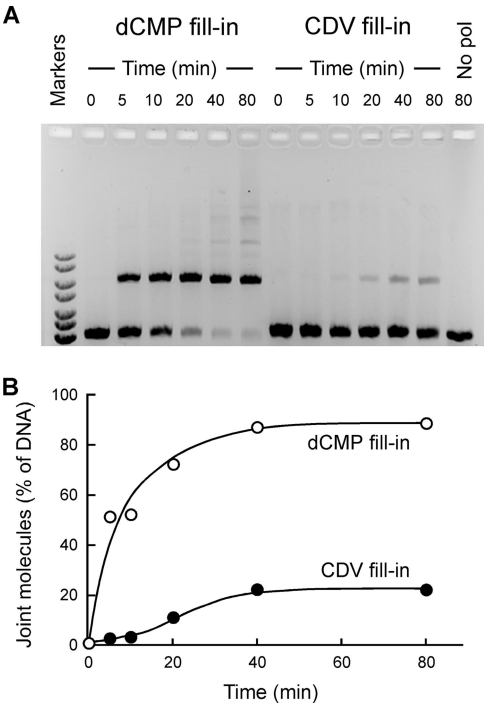

FIG. 4. Effect of CDV on VAC DNA polymerase-catalyzed formation of recombinant molecules in vitro. (A) Duplex strand-joining reactions catalyzed by wild-type VAC DNA polymerase. Each reaction mixture contained 2.5 ng/μl VAC DNA polymerase, 25 μg/ml of VAC single-stranded DNA-binding protein, and 600 ng of pBluescript substrate filled in with dCTP or CDVpp (300 ng cut with XhoI plus 300 ng cut with SpeI). The reaction products were sampled at the indicated times, separated by electrophoresis, and stained with ethidium bromide. The last lane shows DNA recovered from a reaction mixture incubated at 37°C but lacking VAC DNA polymerase (No pol). (B) Quantitative analysis of the reaction products shown in panel A. The distribution of the ethidium fluorescence was used to determine the proportion of DNA migrating as dimers plus higher-order multimeric species ("joint molecules").

idue, which can be used to direct the incorporation of CDV or dCMP (as a control) into the ends of the molecule (Fig. 3, top). We then used VAC DNA polymerase to fill in the ends of the DNA using a mixture of dGTP, dATP, dTTP, and either dCTP or CDVpp. In order to demonstrate what kinds of end structures are generated using this approach, we prepared a parallel set of reactions in which we substituted ^{32}P-labeled oligonucleotide substrates for the linearized pBluescript DNA. These substrates contain ends identical to those produced using XhoI or SpeI digestion of a plasmid DNA, but the small size of the oligonucleotides permits high-resolution analysis of the reaction products on sequencing gels. As we have noted previously (35), VAC DNA polymerase used the four ordinary dNTPs to generate a mix of extension products that terminated 0 to 3 nt away from the 5′ end of the template strand, whereas the products produced in the presence of CDVpp terminated at a site located 1 nt past the site where CDV was incorporated (Fig. 3). No products smaller than the 18-nt primer strand were detected, suggesting that little exonuclease attack occurs under these conditions.

Effect of CDV on linear-molecule recombination in vitro. To test what effect these modifications might have on the duplex fusion reactions catalyzed by VAC DNA polymerase in vitro, we incubated the "end-filled" pBluescript DNAs with E9 polymerase and VAC single-stranded DNA-binding protein in a buffer containing $MgCl_2$ (61). When the control substrates were used in these reactions (i.e., DNA filled in using dCTP), they were first rapidly recombined into 6-kbp dimers and then chased into higher-order concatemers later in the reaction (Fig. 4A). In contrast, very few recombinant molecules were formed in reactions with substrates that had been end filled using CDVpp. Using densitometry, we determined that control reactions produced four- to fivefold more concatemeric DNA than did the reactions using CDV-containing DNAs (Fig. 4B). These results show that incorporating CDV residues into the ends of linear substrates strongly inhibits E9-catalyzed formation of recombinant molecules in vitro.

Effect of CDV on linear-molecule recombination in vivo. We next tested what effect CDV might have on recombination between two linear molecules in vivo. To do this, we digested the luciferase-encoding plasmid pRP406 with either XhoI or AflIII, generating two linear DNA duplexes that share extensive homology and, again, bear ends that can be filled in with CDVpp or dCTP as described above (Fig. 3 and 5A). To show that VAC can efficiently recombine these molecules in vivo, we transfected infected cells with different combinations of cut, but not filled-in, DNAs and then used Southern blotting to characterize the products. Additional dishes of VAC-infected cells were transfected with an equal amount of uncut pRP406, and these cells were used to define 100% recombinant fragments. The cells transfected with two different linear DNAs generated ~34% recombinant restriction fragments relative to those from cells transfected with circular pRP406, whereas only ~2% recombinants were detected in cells transfected with only XhoI-cut DNA, and no recombinants were detectable in cells transfected with AflIII-cut molecules (Fig. 5B). It should be noted that the DNAs defined as being "recombinant" re-

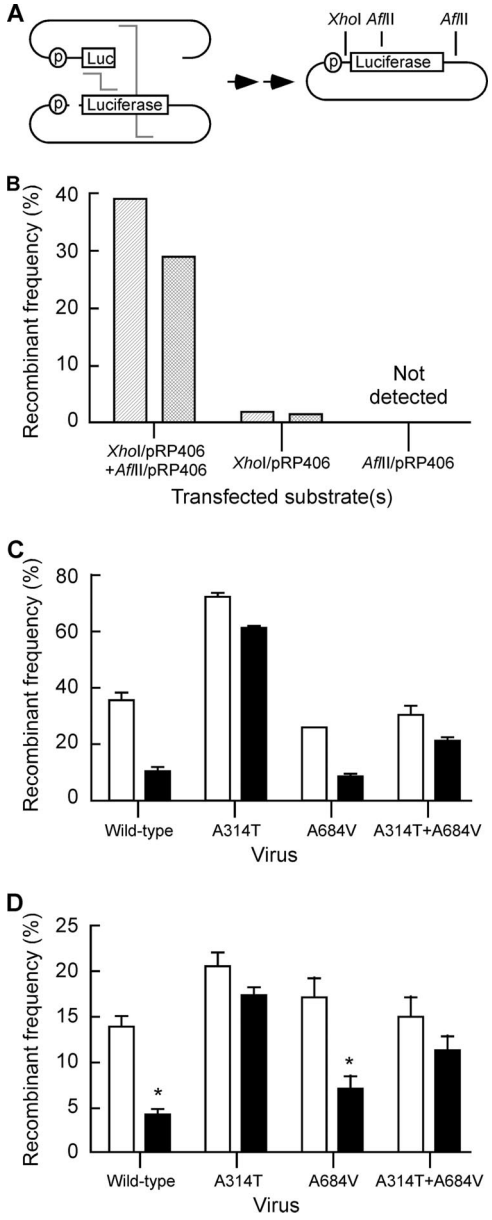

FIG. 5. Effect of CDV on linear-molecule recombination in VAC-infected cells. (A) Substrates and assay used to detect VAC-catalyzed in vivo recombination. Cutting pRP406 with XhoI separates the P11K poxvirus promoter from the luciferase ORF. Cutting pRP406 with AflIII creates two fragments, one of which carries the P11K promoter and the first ~600 bp of the luciferase ORF. This larger fragment was gel purified and used, along with XhoI-cut pRP406, in recombination experiments. (B) Southern blot analysis of DNAs recovered from cells infected with wild-type VAC and transfected with a mixture of XhoI-cut and AflIII-cut pRP406 recombination substrates. The DNA was isolated and processed, and the yield of recombinant molecules was determined as explained in the legend to Fig. 2. The bar graph shows the results of two independent experiments. (C) Southern blot analysis of DNAs recovered from cells infected with mutant and wild-type viruses. The cells were transfected with a mixture of XhoI-cut and AflIII-cut pRP406 recombination substrates that had also been filled in using dCTP (open bars) or CDVpp (filled bars) on the 3′-ended strands. The samples were processed as for panel B. The bar graph shows the results (mean Rf + standard deviation) of two independent experiments. (D) Luciferase-based detection of linear-molecule recombination. The cells were infected with the indicated VAC strains, transfected with substrates that had been filled in using dCTP (open bars) or CDVpp (filled bars), and assayed for luciferase 6 h after infection. The bar graph shows mean Rfs (+SEs) derived from four independent experiments. Asterisks indicate statistically significant ($P < 0.05$) differences between control and CDV treatments for a particular strain. When only one of the two substrates was transfected into VAC-infected cells, it yielded a background Rf of <0.5% (XhoI-cut substrate) or <0.01% (AflIII-cut substrate). Note that for cells infected with the A314T virus, while more recombinants appeared to be detected in the absence of CDV (C and D), we could not show that this difference was statistically significant where such an analysis could be appropriately applied (D).

action products could also be generated by ligation in cells transfected with XhoI-cut pRP406 alone, whereas this is not possible in cells transfected with AflIII-cut DNA alone. Thus, we presume that ligation reactions produce a background of ~2% "recombinants" in these assays and that the remainder of the products are generated via a repair process requiring two molecules with overlapping homology.

We then used the same transfection and Southern blotting methods to examine what effect filling in the ends of the DNAs with CDV had on these reactions. The results are shown in Fig. 5C. Filling in the ends of these substrates with ordinary dNMPs had no obvious effect, relative to the results for DNAs bearing recessed ends, on the Rf in cells infected with wild-type virus (compare Fig. 5B and C). However, CDV incorporation reduced the Rf in cells infected with wild-type viruses ~3-fold, from 36% to 11%. A similar threefold effect was seen in cells infected with a virus encoding the A684V mutation, where CDV reduced the Rf from 26% to 9%. In striking contrast, viruses encoding the A314T mutation exhibited CDV^r recombination, with only small, 1.2- and 1.4-fold reductions in Rfs for viruses encoding the A314T substitution alone or in combination with A684V, respectively.

We also used luciferase-based assays to confirm the trends that we observed with the Southern blotting experiments described above. Although the absolute Rfs calculated from these experiments were consistently lower than those measured by Southern blotting, the trends were essentially identical (Fig. 5D). When DNAs were filled in using ordinary dNMPs and cotransfected into VAC-infected cells, they were recombined efficiently by wild-type and CDV^r strains, producing Rfs of 14 to 20% (Fig. 5D). When CDV was incorporated into the ends of these molecules, the Rfs were significantly ($P < 0.05$) reduced in cells infected with wild-type viruses and in those infected with viruses encoding the A684V substitution alone (from 14 to 4% and from 17 to 7%, respectively [Fig. 5D]). In contrast, there was no significant difference ($P > 0.05$) between the Rfs of control and CDV-containing substrates in cells infected with viruses encoding the A314T substitution either alone or together with A684V (Fig. 5D). Collectively, these results suggest that CDV is an inhibitor of poxvirus recombination but that viruses encoding a mutation in the exonuclease domain are selectively protected from this inhibition.

Effect of CDV on exonuclease and duplex-joining reactions catalyzed by a mutant E9 enzyme in vitro. These experiments suggested that there was some intrinsic property of polymerases encoding the A314T substitution that minimized the

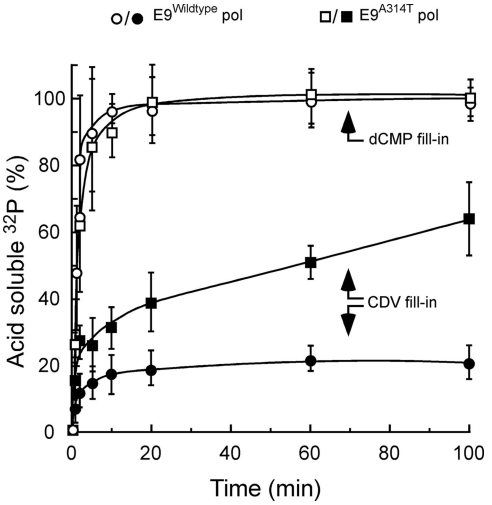

FIG. 6. Effects of CDV on the 3′-to-5′ exonuclease activities of mutant and wild-type VAC DNA polymerases. The pBluescript plasmid was cut with XhoI, and then the ends were filled in with ^{32}P-labeled dTMP plus dCTP (open symbols) or ^{32}P-labeled dTMP plus CDVpp (filled symbols) as described in Materials and Methods. This procedure creates substrates from which the CDV or dCMP residues must be excised before the [α-^{32}P]dTMP label can be attacked. These DNAs (300 ng) were then incubated with 100 ng of either wild-type (circles) or A314T mutant (squares) DNA polymerase. The reactions were stopped at the indicated times; the reaction products were sampled; and the samples were then assayed for acid-soluble ^{32}P. Mean acid-soluble ^{32}P fractions (expressed as percentages) for three independent experiments are shown. Error bars, 95% confidence intervals.

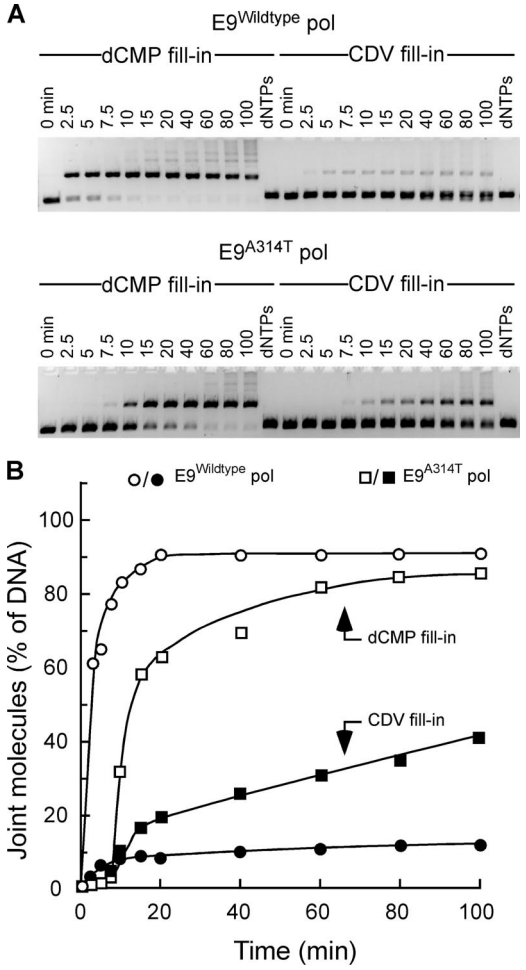

FIG. 7. Effects of CDV on joint-molecule formation catalyzed in vitro by wild-type and A314T-encoding mutant DNA polymerases. (A) Strand-joining reactions were prepared and analyzed as described in the legend to Fig. 4 except that these reactions used either wild-type or A314T mutant DNA polymerases. In some cases, all four dNTPs were added to the reaction mixtures (at 100 μM each) and were incubated for 100 min along with the enzyme and substrates (lanes dNTPs). (B) Quantitative analysis of the reaction products shown in panel A. The distribution of the fluorescent signal was used to determine the proportion of DNA migrating as dimers plus higher-order multimeric species ("joint molecules").

effect of CDV on recombination in vivo. One obvious explanation was that this substitution could alter the 3′-to-5′ exonuclease activity of the enzyme such that CDV residues could be removed from the primer strand and thus create substrates for SSA reactions after further exonucleolytic processing by the polymerase. In order to test this hypothesis, we purified the A314T-encoding mutant form of the E9 enzyme from infected cells. We then used wild-type VAC DNA polymerase to fill in the ends of XhoI-cut pBluescript DNAs in reaction mixtures containing dGTP, dATP, [α-^{32}P]dTTP, and dCTP or CDVpp. The inclusion of [α-^{32}P]dTTP in these reactions led to the incorporation of a ^{32}P-labeled tag on the 5′ side of the site, where dCMP or CDV is incorporated (see Fig. 3). These substrates were then incubated with equal amounts of wild-type or mutant VAC DNA polymerase protein, and the amount of acid-soluble radioactivity released by 3′-to-5′ exonuclease activity was measured (Fig. 6). When the ^{32}P-labeled dTMP was located on the 5′ side of a dCMP residue, both enzymes excised ~100% of the label within 20 min. However, the two enzymes exhibited very different excision kinetics when the label was located on the 5′ side of a CDV residue. After 100 min of incubation, the mutant enzyme was able to solubilize ~64% of the label, while the wild-type enzyme was able to release only ~20% of the label, from these DNAs. The mutant enzyme has clearly acquired the capacity to efficiently excise CDV from DNA ends.

We next tested if the A314T mutant enzyme can overcome the inhibitory effects of CDV on strand-joining reactions catalyzed by the wild-type enzyme in vitro (Fig. 4). As expected, the wild-type enzyme efficiently converted >90% of the dCMP-containing control substrates into dimeric and higher-order joint molecules but yielded a limit of only ~13% joint molecules after 100 min when CDV-containing substrates were used in the reaction (Fig. 7A and B). The mutant enzyme also catalyzed strand joining when presented with dCMP-containing control substrates, although some initial delay in the appearance and rate of production of concatemers was seen. However, the most striking difference between the two enzymes was that the mutant enzyme continued to catalyze strand joining in reactions supplemented with CDV-containing DNA, yielding 3.5-fold more joint molecules than the wild-type enzyme by the end of the 100-min incubation period (Fig. 7B). Importantly, no recombinant molecules were formed in wild-type or mutant reactions when high concentrations (0.4 mM

total) of dNTPs were included (Fig. 7A), demonstrating that inhibition of proofreading activity by high dNTP concentrations also ablates joint-molecule formation, as we have shown previously (22). Collectively, these studies suggest that the capacity of A314T-encoding polymerases to excise CDV from DNA renders virus-catalyzed recombination reactions resistant to CDV during infection.

Other in vivo modulators of poxvirus recombination. These and other studies (63, 64) suggest that poxviruses use the DNA polymerase's 3′-to-5′ exonuclease to promote genetic recombination through SSA reactions in infected cells. This led us to predict that at least two other factors may play additional roles in virus recombination. First, the activity of E9's exonuclease should be modulated by the concentration of dNTPs, since an abundance of dNTPs would favor DNA synthesis over exonucleolytic strand-processing reactions (22). Decreasing the availability of dNTPs should stabilize single-stranded gaps and favor recombination. Second, single-stranded DNA-binding proteins play an important role in many recombination reactions (7, 46), and we have previously shown that this is also true of E9-catalyzed strand-joining reactions in vitro (61). In particular, the product of the VAC early gene *I3L* is a high-affinity single-stranded DNA-binding protein (47, 53) that stimulates strand joining in vitro (61). We therefore decided to test the two predictions that altering the levels of dNTPs and I3 protein should also affect virus recombination in vivo.

In order to test what role dNTPs might play in modulating virus-promoted recombination, we examined the effect of the ribonucleotide diphosphate reductase inhibitor HU on recombination rates in infected cells. HU inhibits both cellular and VAC-encoded ribonucleotide reductases and thus causes a reduction in the dNTP pools by inhibiting the upstream conversion of rNDPs to dNDPs (50). We transfected VAC-infected cells with our luciferase-encoding linearized recombination substrates and then replaced the medium with fresh media containing different concentrations of HU at 4 h postinfection. The amount of recombination was then determined by luciferase assays performed after another 4 h of incubation. By using a similar method to treat VAC-infected BSC-40 cells with HU, it has been observed that 0.5 mM HU will deplete the dNTP pools by 50% for dGTP/dCTP and by 90% for dATP within 1 h of addition of HU to the medium (50). At HU concentrations of 0.5 mM and higher, we observed a significant ($P < 0.05$) increase in the mean (±SE) Rf, from 37% ± 5% in drug-free medium to 69% ± 2% and 68% ± 3% in media containing 0.5 and 5 mM HU, respectively (Fig. 8). Although no statistically significant differences in the Rf were detected at lower HU concentrations, a consistent trend was still observed, with the Rf increasing as the HU dose increased (Fig. 8), suggesting that HU treatment could enhance recombination rates in VAC-infected cells.

In order to test whether the VAC single-stranded DNA-binding protein I3 served any role in virus recombination, we used siRNA technology. Previous attempts to study the presumptive role of I3 in viral replication were hindered by the inability to delete the *I3L* gene from the viral genome, suggesting that this highly conserved poxvirus gene is essential (47). Using a siRNA targeting I3 mRNA (*I3L*-4), we were able to reduce I3 protein levels by 65 to 85%, relative to levels in cells that were not transfected with siRNA (Fig. 9A). The specificity of the method was demonstrated by using a control siRNA that had no effect on I3 levels (Fig. 9A). Using these conditions, we then investigated the effect of I3 knockdown on virus recombination by using transfected, luciferase-encoding linear DNA substrates. We found that reducing the level of I3 also reduced the Rf to ~50% of that detected in cells not receiving siRNA or in cells transfected with the control siRNA (Fig. 9B). We also found that I3 knockdown inhibited viral DNA replication (Fig. 9C), suggesting that I3 is also required for genome replication. These results show that VAC recombination and replication reactions utilize I3 in some capacity, as we would predict, although the precise role of I3 in these reactions requires further investigation.

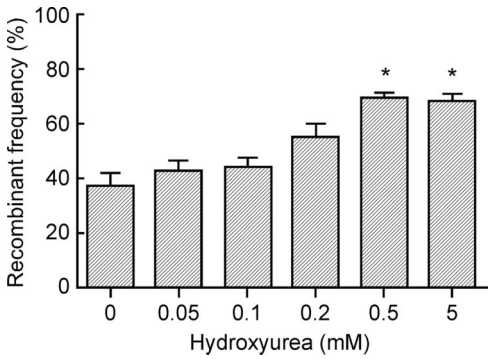

FIG. 8. Effect of HU on recombination in VAC-infected cells. XhoI-cut and AflIII-cut pRP406 recombination substrates were transfected into cells infected with wild-type VAC. The culture medium was replaced with fresh medium alone or with fresh media containing the indicated concentrations of HU at 4 h postinfection. Protein extracts were prepared at 8 h postinfection and were assayed for luciferase. Each bar represents the mean (+SE) Rf, expressed as a percentage of the luciferase signal detected in cells transfected with uncut pRP406, from four independent experiments. Asterisks indicate that the Rf measured in treated cells differed significantly ($P < 0.05$) from that measured in untreated controls.

DISCUSSION

Although Fenner and Comben first described recombination between coinfecting poxviruses 50 years ago (16), the enzyme(s) that catalyzes this process and the mechanism remain obscure. Our previous studies had suggested that poxvirus recombination reactions used some form of SSA mechanism but that these reactions exhibit the unusual property of using a 3′-to-5′ exonuclease to resect duplex ends (63, 64). VAC encodes only one known exonuclease, and that is the 3′-to-5′ exonuclease of the viral DNA polymerase. When these observations are combined with evidence that highly purified VAC E9 can catalyze duplex-DNA-joining reactions in vitro and is seemingly required for recombination in vivo, they provide strong support for the hypothesis that poxviruses use the DNA polymerase proofreading activity to catalyze genetic exchange.

Mutant viruses provide an ideal way of testing this hypothesis. However, our attempts to rescue mutant VAC strains lacking the 3′-to-5′ exonuclease function were unsuccessful. Although two temperature-sensitive *E9L* alleles serve as very useful selectable markers, one sees a strong selection against coconversion of these sites and three sites encoding aspartic

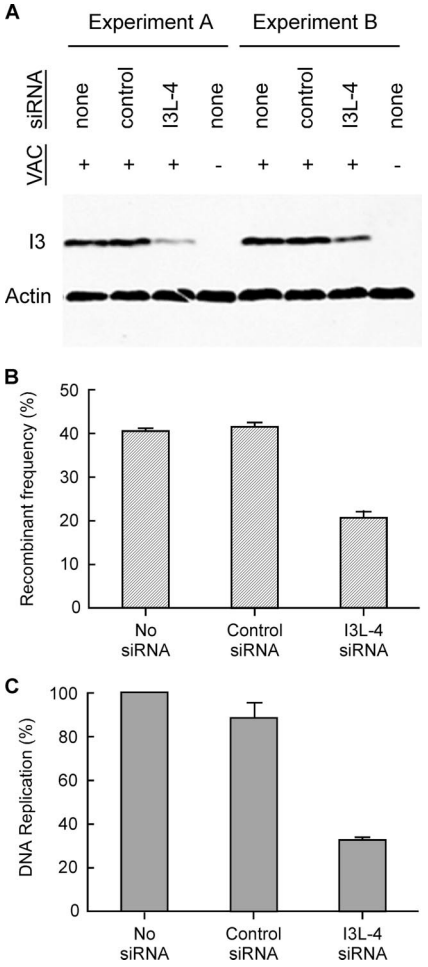

FIG. 9. A siRNA targeting the VAC-encoded single-stranded DNA-binding protein (I3) inhibits virus recombination. (A) Western blot analysis showing reduction of I3 protein levels by siRNA. Cells were first infected (or mock infected) with wild-type VAC and then cotransfected with a mixture of XhoI-cut and AflIII-cut pRP406 recombination substrates plus the indicated siRNA, targeting I3 mRNA transcripts (*I3L*-4) or an unrelated sequence (control), or no siRNA ("none"). Cell-free lysates were prepared at 8 h postinfection, and Western blotting was performed using antibodies directed against I3 and cellular β-actin. (B) Effect of an I3-targeted siRNA on VAC recombination. Cells from another set of dishes were harvested in parallel and assayed for luciferase. Each bar represents the mean Rf (expressed as a percentage) from three independent experiments. Error bars, SEs. (C) Effect of an I3-targeted siRNA on VAC replication. Each bar represents the mean viral DNA quantitation value (expressed as a percentage of the value for the "no-siRNA" treatment, taken as 100%) determined by slot blot analysis. Error bars, standard deviations.

acid residues that should be critical for exonuclease function. This is illustrated by a dramatic reduction in the recovery of recombinant virus (Fig. 1B), which we presume is due to the loss of recombinant viruses encoding exonuclease mutations. This bias is exacerbated as the linkage gets tighter, presumably due to the reduced likelihood of recombination separating the two markers. The strongly biased selection against the coconversion of these markers is illustrated by the fact that although the D166A and D*ts*83 (H185Y) mutations are located only ~60 bp apart, none of the temperature-resistant viruses encoded a novel AluI site that is diagnostic for the D166A substitution (Fig. 1C). The fact that other silent mutations can be introduced into the sites encoding Exo II and III motifs shows that these effects are not caused by some peculiar impediment to recombination (Fig. 1D and F). The selection pressure specifically disfavors the production of viruses encoding D-to-A substitutions at sites predicted to be critical for exonuclease activity, and this leads us to conclude that the 3′-to-5′ proofreading exonuclease is an essential virus function.

Because one cannot generate a VAC strain constitutively lacking 3′-to-5′ exonuclease activity, we decided to use an alternative, antiviral-based approach to study the links between proofreading and recombination. As a starting point, we determined that when CDV is added to the culture medium, it inhibits recombination between circular plasmid substrates in cells infected with wild-type VAC (Fig. 2). Plasmid substrates are useful tools for studying poxvirus replication and recombination, because the DNA accumulates at sites of viral replication in the cytoplasm of infected cells, and plasmid replication utilizes all five of the virus-encoded proteins needed for viral genome replication (12). A previous report has shown that treating VAC-infected cells with DNA polymerase inhibitors such as cytosine arabinoside and aphidicolin also inhibits the recombination of transfected plasmid substrates, emphasizing the need for a functional E9 in VAC recombination (6). Furthermore, this study found that the rate of recombination of these plasmid substrates by different mutant VAC strains was independent of the rate of replication of these substrates by each strain (6). This suggested that the VAC DNA polymerase was participating in homologous recombination primarily at some level other than just DNA synthesis (6). CDV also inhibited plasmid recombination, but the amount of recombination was affected differently by different CDV^r mutations (Fig. 2B). Viruses encoding the A314T mutation were less susceptible to CDV than A684V-encoding or wild-type viruses (Fig. 2B), even when the drug doses were chosen to have comparable effects on DNA replication and virus yield. Although one can never exclude the possibility that the different drug doses are in some unknown way modulating host effects on virus recombination, the simplest conclusion that can be drawn from these experiments is that the 3′-to-5′ exonuclease activity likely serves a different role in virus recombination than does the 5′-to-3′ polymerase activity.

To avoid any impact of these hypothetical host effects and further elucidate the effect of CDV on poxvirus recombination, we prepared linear recombination substrates containing CDV as the penultimate residue on the 3′ ends (Fig. 3). DNAs bearing these structures are resistant to exonuclease attack (34). This alteration clearly inhibited joint-molecule formation catalyzed by wild-type E9 in vitro (Fig. 4), as well as recombinant production in cells infected with wild-type and A684V-encoding VAC strains in vivo (Fig. 5). In contrast, a mutant E9 protein incorporating the A314T substitution could still catalyze joint-molecule formation in vitro (Fig. 7), and viruses encoding the A314T mutations still efficiently catalyzed the recombination of CDV-containing substrates in vivo (Fig. 5). Biochemical studies showed that these effects correlate with an enhanced capacity of the A314T-encoding E9 enzyme to excise CDV from DNA (Fig. 6). A complicating factor is that one can never completely fill in the ends of these substrates with CDV.

Using densitometry, we estimated that ~17% of the XhoI-cut substrates that were end filled with CDV, shown in Fig. 3, lacked 3′ CDV residues. This finding corresponds well with the 13 to 20% yield of recombinant molecules typically produced by wild-type E9 using CDV-bearing substrates (Fig. 4A and 7A). Of course, these limitations in end-filling efficiency could also explain why some recombination was still detected in cells infected with wild-type and A684V-encoding viruses and transfected with substrates into which CDV had been incorporated (Fig. 5).

The 2- to 3.5-fold differences in the absolute recombination frequencies between our luciferase (Fig. 5D) and Southern blot-based (Fig. 5C) assays should be noted. Southern blotting represents the method of choice for providing insights into the structure and quantity of the DNA recovered from infected and transfected cells. However, these techniques are subject to high experimental variation due to possible differences in infection and transfection efficiency. Luciferase-based assays are convenient and are normalized to β-galactosidase expression from a cotransfected plasmid, thus providing more control for infection/transfection variations and differences in plasmid DNA replication. However, these assays tend to underestimate the Rf, because a functional luciferase can be expressed only after substrate recombination has created an intact gene and transcription and translation has occurred. Despite these differences, both methods provided a consistent finding: CDV residues inhibit recombination, and viruses encoding the A314T substitution are far more resistant than other strains.

The enhanced ability of A314T-encoding E9 to excise CDV from DNA ends likely explains why this mutation arose during repeated passage of VAC in CDV-containing media (1). Interestingly, the same mutation is recovered when VAC is passaged in media containing the related compound (*S*)-9-[3-hydroxy-2-(phosphonomethoxy)propyl]adenine (HPMPA) (1). We have also found that HPMPA, when located in the penultimate position in a primer strand, is resistant to VAC E9 proofreading activity (34) and that HPMPA residues also block joint-molecule formation in vitro (D. Gammon, unpublished data). The A314T mutation is the first and most common mutation recovered when poxviruses are exposed to nucleoside phosphonate drugs, and the strong selection pressure acting at this site suggests that exonuclease activity is essential either because of a need to excise drugs that would otherwise inhibit replication or because of a requirement for gapped DNA molecules in some process such as viral recombination, or both. We have previously suggested that the A314 residue would likely be located in a β-hairpin structure that is highly conserved among B-family DNA polymerases (1, 24). A recent study of RB69 and T4 phage DNA polymerases either containing mutations in this structure or lacking the β-hairpin structure completely have suggested that the β-hairpin plays an important role in separating primer and template strands and thus facilitates the removal of mismatched bases (24, 52). RB69 polymerase mutants lacking the β-hairpin can degrade single-stranded DNA, as well as duplex DNA containing three terminal mismatches, as efficiently as the wild-type enzyme but are hindered in their ability to excise a single 3′-terminal mismatched nucleotide (52). We have noted that 3′-terminal CDV and HPMPA molecules are as readily excised by wild-type E9 as are dCMP and dAMP (34, 35). However, the incorporation of one additional nucleotide renders CDV and HPMPA residues quite resistant to the exonuclease. We suspect that the A314T substitution confers on VAC E9 an enhanced ability to destabilize a strand bearing a nucleoside phosphonate drug in the penultimate 3′ position, leading to strand separation, excision of the drug residue, and 3′-to-5′ resection of duplex DNA. These resected molecules could then serve as recombination substrates in an annealing reaction stimulated by the VAC single-strand binding protein I3. We have been shown previously that I3 promotes Mg^{2+}-dependent DNA aggregation in vitro (53) as well as increasing the efficiency of joint-molecule formation in reactions catalyzed by purified VAC DNA polymerase (61). Single-strand DNA-binding proteins are known to play an important role in many repair, recombination, and replication systems. The fact that one can inhibit in vivo recombination with a siRNA targeting I3 mRNA (Fig. 9B) is consistent with the protein also serving some recombination-related function(s) in VAC-infected cells. However, knocking down I3 levels also inhibits DNA replication (Fig. 9C), an important fact that is discussed in more detail below.

If a viral DNA polymerase plays some role in catalyzing recombination, it follows that any intervention that causes a switch between polymerization and exonuclease activities should also alter recombination rates. We have previously noted that increased dNTP concentrations inhibit E9-catalyzed strand-joining reactions in vitro (22). To determine whether dNTP pools also affected recombination in vivo, we examined the effects of the ribonucleotide reductase inhibitor HU on VAC recombination. HU has previously been shown to inhibit VAC replication (50), and at 10 mM concentrations, it can also enhance the recombination of circular plasmids in VAC-infected cells (6). HU has also been shown to enhance cellular recombination rates (19, 28, 32). We varied the drug dose and detected a clear trend where the yield of recombinants increased with increasing doses of HU (Fig. 8). The highest doses of HU that were tested (0.5 and 5 mM) are known to reduce dATP pools to about 10% of the levels detected in untreated, VAC-infected BSC-40 cells (50), and they increased recombinant production ~2-fold in our studies. Of course, these experiments assume that HU exclusively inhibits a ribonucleotide reductase activity in VAC-infected cells and does not induce some other, unknown host-dependent recombination-enhancing effect(s). To test this, we have recently generated a VAC strain lacking the *F4L* gene, which encodes the small subunit of the viral ribonucleotide reductase. This Δ*F4L* virus also exhibits an enhanced-recombination phenotype linked to deficiencies in replication. In particular, this virus recombines transfected linear plasmid DNAs at frequencies ~12% higher than wild-type VAC while producing only ~20% of the DNA seen in cells infected with wild-type virus (D. Gammon, unpublished data).

How one interprets all of these experiments is frustratingly complicated by the intimate links between viral recombination and replication. Where possible, we have used cotransfected plasmids, encoding β-galactosidase, to normalize the levels of luciferase and thus avoid detecting a simple artifactual link between reduced rounds of replication and reduced amounts of replication-associated recombination. Moreover, it should be noted that these experiments show how VAC replication and recombination are not always linked in such a simple

manner. Knocking down I3 expression inhibits replication and inhibits recombination (Fig. 9), but interfering with the ribonucleotide reductase activity inhibits replication (50) while enhancing recombination (Fig. 8). Furthermore, a previous study of the recombination of transfected plasmid substrates in cells infected with mutant VAC found that the recombination rates measured in these different strains were largely independent of the plasmid DNA replication rates (6). VAC replication and recombination are undoubtedly tightly linked processes, sharing many common enzymes, but one cannot explain these observations with a simple model that directly links replication rates to recombination frequencies.

Our studies suggest a more complex mechanism by which a DNA polymerase could catalyze recombinational repair in a manner dependent on dNTP regulation of the balance between 5′-to-3′ DNA synthesis and 3′-to-5′ exonucleolytic processing. Poxviruses have recently been shown to encode a DNA primase (11), suggesting that DNA replication involves leading- and lagging-strand DNA synthesis at a classical replication fork. Under such circumstances, a potentially lethal double-strand break would be created following a collision between the replication fork and a nick located on either strand ahead of the replication complex (Fig. 10A). By attacking the 3′-ended strand, the 3′-to-5′ proofreading exonuclease could expose sufficient homology to permit the reformation of the original replication fork through I3-assisted SSA. VAC DNA polymerase and DNA ligases could then repair the nick or small gap.

It is more difficult to speculate on how this process might be regulated, but several unusual features of poxvirus biology suggest a way in which dNTP concentrations could play an important role in regulating DNA synthesis versus degradation. The VAC-encoded ribonucleotide reductase (comprising the *I4L* and *F4L* gene products) plays a key role in the biosynthesis of the extraordinary amounts of dNTPs required for viral replication (26). Interestingly, F4 interacts with I3 (9), and it has been suggested that the VAC single-stranded DNA-binding protein I3, like the T4 bacteriophage gp32 (57), can recruit a putative "dNTP synthetase complex" to the replication fork and thus help couple dNTP production and consumption (26). If this were true, the collapse of a replication fork would disturb this equilibrium, and the resulting change in the dNTP microenvironment could perhaps favor strand gapping over DNA synthesis (Fig. 10B). To date we have used immunoprecipitation methods to confirm that I3 can interact with F4 in vitro (C. Irwin and D. Evans, unpublished data), but further studies are needed to test this hypothesis.

Our model provides important new insights into the diversity of enzymes and mechanisms that can be used to catalyze double-stranded break repair. This process can be partitioned into pre- and postsynaptic events, and the role that DNA polymerases might play in it reflects the multiplicity of reactions potentially catalyzed by DNA polymerases. It is well established that particular DNA polymerases can play a specialized role in catalyzing the postsynaptic DNA synthesis associated with homologous (25, 33, 39, 40) and nonhomologous (4, 5) recombination. It has also been shown that, in yeast, Pol2 proofreading activity can catalyze the postsynaptic processing of imperfect recombinant intermediates (54) in a manner similar to that of the reactions that we have shown are catalyzed by

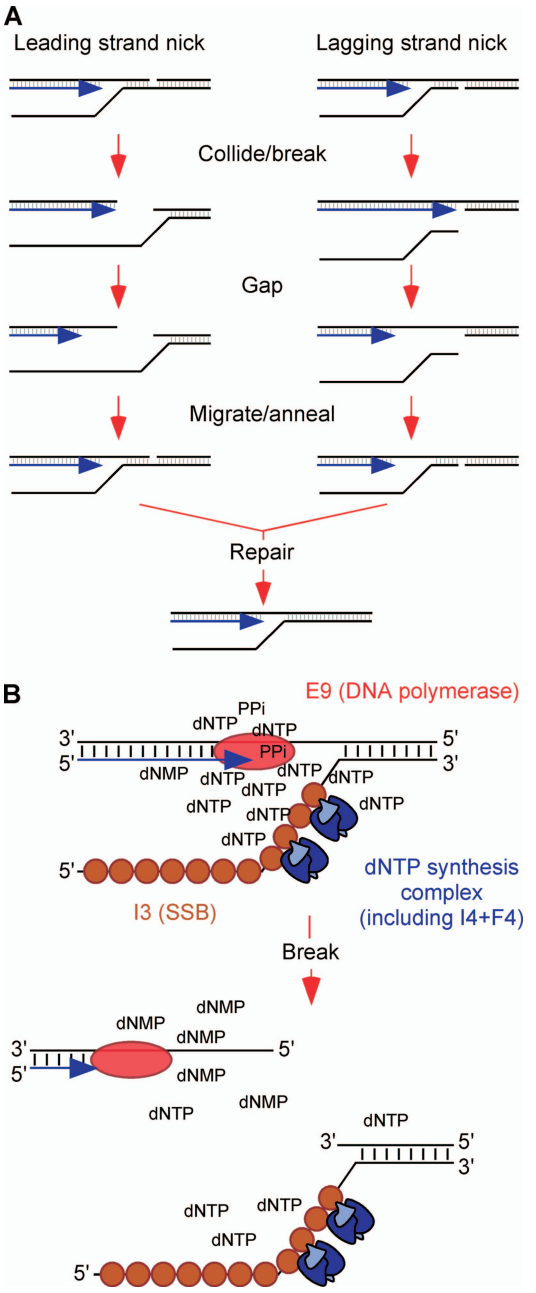

FIG. 10. Proposed model for poxvirus recombination and its regulation by dNTPs. (A) Diagram of how a collision between the viral replication fork and a preexisting nick will lead to replication fork collapse. We propose that the E9-encoded 3′-to-5′ exonuclease can attack the newly synthesized strand (blue arrowhead), creating a 5′-ended single-stranded tail that can then be used to reassemble the fork through SSA. This process could create a futile cycle of breakage and reannealing (not shown) that would continue until the break has been repaired and sealed. Replication could then proceed normally. (B) Diagram of how such a scheme could be regulated by dNTPs. We suggest that poxviruses might use the single-stranded DNA-binding protein I3 (brown circles) to recruit a putative dNTP synthetase complex (blue) to the replication fork and thus closely couple dNTP biogenesis with dNTP consumption. Disruption of this structure through strand breakage and spatial separation of the dNTP source from its sink could reduce dNTP availability, inducing the DNA polymerase (red oval) to switch into a proofreading mode that catalyzes recombinational repair.

the E9 3′-to-5′ exonuclease (21). However, our present studies strongly suggest that VAC can also use the E9 proofreading activity to catalyze a presynaptic step in genetic exchange. It is difficult to prove this with certainty, because one can always suggest more-complex ways in which the enzyme's role might be limited to catalyzing just a postsynaptic step(s) in recombination. For example, we cannot absolutely rule out the possibility that the proofreading activity of E9 is used to remove CDV from the 3′ ends of the DNA strands after they have entered into a process such as a synthesis-dependent strand-annealing (SDSA) reaction. SDSA reactions typically require the presynaptic 5′-to-3′ gapping of recombination substrates, which exposes 3′-ended single-stranded DNAs that can then invade duplex strands, form displacement loops, and prime DNA synthesis (43). However, this model seems unlikely, because if poxviruses did use SDSA reactions in vivo, it would follow that these viruses should very efficiently catalyze recombination between linear duplex and circular substrates (51), and they clearly cannot (64). Furthermore, we have previously examined the fate of mismatch-tagged recombination substrates and demonstrated that >80% of the mature recombinants recovered from VAC-infected cells retain the mismatched nucleotide originally located on the 5′ strand (63, 64). This shows that double-stranded breaks are processed in a 3′-to-5′ manner during virus recombination, which would be inconsistent at least with standard SDSA models. We suggest that the in vitro biochemical data provide the simplest and most logical explanation for the in vivo data and that one need posit no more complex a model than that the *E9L*-encoded proofreading activity also catalyzes presynaptic gapping in VAC recombination. This proposal does differentiate the VAC recombination system from the vast majority of SSA reactions that depend on 5′-to-3′ presynaptic processing by simple exonucleases (e.g., those of herpesviruses [44], yeast [42], and mammals [7]). Whether this unique mechanism reflects some special constraints created by poxvirus biology (e.g., cytoplasmic replication, genome size, the mechanism of dNTP biogenesis, the lack of a RecA-like recombinase), some unusual feature(s) of E9, or the first example of what is actually a more widespread biological process remains a very interesting question.

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